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Drug Discovery

Biogenic synthesis of silver nanoparticles and their antioxidant and antibacterial activity aqueous leaf extracts of *Centella asiatica* Linn.

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ABSTRACT

The present study, a simple and eco-friendly, biosynthesis of silver nanoparticles using the plant leaves of *Centella asiatica*. In this study, AgNPs were synthesized extra cellular by *Centella asiatica* at room temperature. The AgNPs were quite stable without using any toxic chemicals as capping agents. The spherical AgNPs ranged in size from 2 nm, and showed promising broad-spectrum antimicrobial activity. The ability to synthesize AgNPs as potential antimicrobial agents using *Centella asiatica* is highly promising for the green, sustainable production of nano-metals, and this enhances its widespread application as an important strategy. Although

Ag NPs themselves do not have any antimicrobial activity, they may act as drug carriers. In other words, because of the presence of Ag NPs, the surface area increases and hence it carries a lot of drug on its surface. Obviously, when the amount of drug in proximity of a bacterium is more, the antibacterial property may be enhanced. Applications of such nanoparticles in medical and other applications make this method potentially useful for the large-scale synthesis of other inorganic nano materials. In our opinion, the mechanism(s) of possible enhancement of the antibacterial activity of conjugates is still an open question and needs further study. The silver nanoparticles were characterized by UV-spectroscopy analysis, FTIR, XRD, SEM and EDX.

Keywords: Silver NanoParticles, Antioxident, Antibacterial activity, *Centella asiatica* Linn.

1. INTRODUCTION

Nanotechnology is fast growing by producing Nano products and nanoparticles that can have novel and size-associated physio-chemical properties differing considerably from larger matter. The new properties of nanoparticles have been subjugated in a broad range of potential applications in cosmetics, renewable energies material, medicine, biomedical devices and environmental remediation (Tran *et al*., 2013). The synthesis of nanoparticles by chemical and physical methods has long been used in the field of nanotechnology (Ahmadi *et al*., 1996)

The biological synthesis of silver nanoparticles is suitable and extracellular method which is environmentally safe. In the present study the silver nanoparticles were synthesized quickly by using the medicinal plants *Centella asiatica* L. against the cancer. Chavicol is present in *Centella asiatica* leaf. It is a phenolic compound and powerful reducing agent against metallic ion reduction (Santosa *et al*., 1994). At present, biosynthesis of SNPs were done using plant extracts of *Centella asiatica* L., although biosynthesis of silver nanoparticles by plants such as *Solanum xanthocarpum* L. *Berry* (Amin *et al*., 2012), *tea leaf* (Loo *et al*., 2012), *Callicarpa maingayi* stem bark (Shameli et al., 2012) *Bauhinia variegate* (Kumar and Yadav., 2012), *Terminalia chebula* (Mohan kumar *et al*., 2012), *Trachyspermum ammi and Papaver somniferum* (Vijayaraghavan *et al*., 2012), *Hevea brasiliensis* (Guidelli *et al*., 2011), *Memecylon edule* (Elavazhagan, Arunachalam., 2011), *Aloe vera* (Panda,Kar.,1998) have already been reported. However, the prospective of the plants as biological materials for the synthesis of nanoparticles is yet to be fully explored.

2. MATERIALS AND METHODS

Collection of Plant material and Identification

The *Centella asiatica* was collected from Tiruppur district, Tamilnadu, India. The plant was identified *Centella asiatica* Dr. SN.Nagarajan at the Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamilnadu, India.

Fig.1 *Centella asiatica (study plant)*

Preparations of the Plant extract

The *Centella asiatica* extract was prepared by taking 20 g of thoroughly washed and finely cut *Centella asiatica i*n a 250 mL Erlenmeyer flask with 100 mL of sterile distilled water and then boiled the mixturefor 10 min. The solution was then removed from the head source and left at room temperature. Following this step the extract was then filtered through a Whatman filter paper No.1. The extract was kept in refrigerator at 4ºC for further experiments.

Qualitative Phytochemical Analysis

Phytochemical components of the aqueous extracts of *Centella asiatica* were screened by using standard methods. The components analyzed were Alkaloids, Flavonoids, Saponins, Tannins, Triterpinoids and Glycosides.

Alkaloids (Raaman, 2006)

Solvent free extract, 50 mg of the plant sample was stirred with one mL of dilute hydrochloric acid and filtered. The filtrate was tested for alkaloids. Mayer's Test: To the filtrate, a drop of Mayer's reagent was added along the sides of the test tube. A white precipitate indicates the test as positive.

Flavonoids (Raaman, 2006)

Alkaline reagent test: Two mL of aqueous solution of the extract was treated with 1 mL of 10 % ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

Saponins (Raaman, 2006)

Fifty mg of the plant sample was ground with 3 mL of distilled water and diluted with the same, made-up to 20ml. The suspension was shaken in a graduated cylinder. After 15 min, a two cm layer of foam indicates the presence of saponins.

Glycosides (Keller-kilani test)

Crude extract was mixed with 2mL of glacial acetic acid containing 1-2 drops of 2% solution of FeCl3. The mixture was then poured into another test tube containing 2mL of concentrated H2SO4. A brown ring at the interphase indicated the presence of cardiac glycosides.

Tannins (Iyengar, 1995)

One mL of water and 1-2 drops of ferric chloride solution was separated and 1 mL of aqueous extract of the plant sample. Blue color was observed for Gallic tannins and green black for catecholic tannins.

Terpinoids (Keller-kilani test)

To 4 mg of the sample was treated with 0.5 mL of acetic anhydride and 0.5mL of chloroform. Concentrated sulphuric acid was added slowly along the sides of the test tube. The presence of red violet colour was observed for terpinoids.

Anti-Bacterial Assay

Bacterial culture

Clinical isolates of microorganisms *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Klebsiella peneumoniae* were obtained from PSG Hospital, Coimbatore.

Preparation of inoculums

A loopful of strain was inoculated in 30 mL of nutrient broth bacteria composition (Dextrose, 4 g; Peptone, 1 g; Distilled water, 100 mL) in an Erlenmeyer flask and incubated on a rotary shaker at 37 oC for 24 h to activate the strain.

Bioassay (Perez *et al.,* 1990)

The antibacterial activity of the leaf extract was determined in accordance with the agar-well diffusion method. Nutrient agar plates were swabbed with a suspension of Staphylococcus *aureus* using sterile cotton swab. Wells of 6 mm were bored with a sterile cork borer in the swabbed plates and filled with the extract. Inoculated plates were incubated uninverted at 37 oC for 24 hrs. Controls were set up in parallel using the solvent that was used to reconstitute the extract. The plates were observed for zones of inhibition after 24 h. The results were compared with the standard antibiotic Neomycin (150 mg mL-1).

***In vitro* antioxidant assay**

Reducing power determination:

Ferric reducing power (FRP) was assessed using the potassium ferric cyanide assay. The reducing power of extract was determined according to the method of Oyaizus, (1986). Different concentration (0.5-3.5ml) of AgNPs solution was mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 5 C for 20 minutes. A portion (2.5ml of

trichloroacetic acid, 10%) was added to the mixture to stop the reaction which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and ferric chloride (0.5ml, 0.1%) and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated reducing power. BHT (Butylhydroxy toluene) was used as positive control.

Biosynthesis of AgNPs from *Centella asiatica*

The aqueous solution of 1mM concentration silver nitrate (AgNO3) was prepared to synthesize silver nanoparticles from *centella asiatica*. For the experiment briefly, 5mL of *centella asiatica* leaves extract was slowly added to 100mL of aqueous solution of 1mM concentration AgNO3 while stirring, for reduction into Ag ions. The formation of dark brown colour was observed after 8 h incubation at room temperature and λ max was taken using SUV-Visible spectroscopy (UV-2600 series shimadzu UV-vis spectrophotometer from 200-800 nm at a resolution of 1nm).

Then the silver nanoparticles solution was purified by repeated centrifugation at 10,000 rpm for 20 min to isolate Ag nanoparticles free from other bioorganic compounds present in the solution. After centrifugation the obtained particles were washed with distilled water for 2 to 3 min and kept it in Hot air oven for drying at 60ºC for 2 h. The effectiveness and accuracy in results without any contamination, each and every steps of the experiment were maintained under sterility conditions.

Characterization techniques

UV-Visible spectroscopy

Formation of silver particles (After 24h incubation at room temperature) was confirmed by the colour change of the solution and the surface plasmon resonance band was obtained by UV-Visible spectral analysis which was done by using UV-Visible spectrophotometer (JASCO, V-670) from 300-700 nm at a resolution of 1 nm.

FTIR- spectrum

Fourier transform infrared spectroscopy (FTIR) analysis of aqueous extract of *Centella asiatica* leaf using FTIR Shimadzu-8400S was carried out at PSG College of Arts and Science, Coimbatore, Tamilnadu, India. The FTIR was recorded in the range of 400 to 4,000 cm-1. The various modes of vibrations were identified and assigned to know the different functional groups present in the extract.

X-ray Diffraction spectrum

X-ray diffraction (XRD) measurement of the green synthesized using *centella asiatica* leaf extract reduced silver particles was carried out using X'Pert Pro X-ray diffractometer (PAN analytical BV, The Netherlands) equipped with Cu/Kα radiation source using Ni as filter at a setting of 30kV/30mA. All X-ray diffraction data were collected under the experimental conditions in the regular angular range.

The crystalline silver nanoparticle was calculated from the width of the XRD peaks, using a Debye-Scherer formula,

$$D = \frac{0.94\lambda}{\beta\cos\theta}$$

Where D is the average crystallite domain size perpendicular to the reflecting planes, λ is the X ray wave length, β is the full width at half maximum and θ is the diffraction angle.

Scanning Electron Microscopy

Each of the colloidal solution containing silver nanoparticles synthesis using *Centella asiatica* leaf extract was centrifuged at 5,000 rpm for 20 min. The supernatants were discarded and the final pellets were dissolved in 1000µL of deionized water. The pellet was mixed properly and carefully placed on a glass cover slip followed by air-drying. The cover slip itself was used during scanning electron microscopy (SEM) analysis. The images of silver nanoparticles were obtained in a scanning electron microscope (Fb-Quanta 200 SEM machine). The details regarding applied voltage, magnification used and size of the contents of the images were implanted on the images itself.

Energy-dispersive X-ray (EDX) analysis

Energy-dispersive X-ray (EDX) analysis referred to as EDS, is an x-ray technique used to identify the elemental composition of materials. The bio reduction synthesized silver nanoparticles using *Centella asiatica* leaf extract subject to the EDX spectrum using Fb-Quanta 200 resolution attached scanning electron microscope to confirm the presence of silver in the particles as well as to detect other elementary compositions of the particle.

3. RESULTS AND DISCUSSION

Phytochemical screening

The preliminary photochemical screening of the aqueous extraction of *Centella asiatica* leaf was reported (Table-I). The positive result for the presence of Saponins, Flavonoids, Steroid, Terpenoids, Phenols, Tannins and substance observed in aqueous extract of *Centella asiatica.*

Antibacterial activity of aqueous leaf *Centella asiatica*

The antibacterial activity against the acetone leaf extract of *Centella asiatica* against pathogenic bacteria's such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Klebsiella peneumoniae* showed varied results (Table -2 &plate-1) .The10mg/mL concentration has shown maximum activity against *Bacillus subtilis* measuring 16mm and minimum against *Escherichia coli* 15mm. The *Klebsiella peneumoniae* and *Staphylococcus aureus* showed 13mm and 11mm. The 40 mg/mL concentration showed maximum activity against *Staphylococcus aureus* (19mm) and minimum activity against *Escherichia coli* (21mm). The other strains showed 18mm and 22mm against *Klebsiella peneumoniae* and *Bacillus subtilis* respectively.

Table 1: Phytochemical pres analysis of aqueous leaf extract of *Centella asiatica*

compounds	Result
Saponins	Positive
Flavonoids	Positive
Steroid	Negative
Terpenoids	Positive
Phenols	Positive
Tannins	Negative

Table 2: Antibacterial activity of aqueous leaf extract of *Centella asiatica*

DOSE	DIAMETER OF INHIBITION ZONE (mm)			
	ORGANISMS			
	E.coli	Bacillus sps.	Staphylococcus	Klebsiella
CONTROL(streptomycin)	15mm	16mm	13mm	11mm
10µl	15mm	15mm	16mm	18mm
20µl	17mm	16mm	17mm	19mm
30µl	19mm	17mm	18mm	20mm
40µl	21mm	18mm	19mm	22mm

Table: 3 Reducing power activity of aqueous leaf extract of *Centella asiatica*

CONCENTRATION	OD at 700nm
Blank	0.00
0.5	0.39
1	0.43
1.5	0.45
2	0.56
2.5	0.58
3	0.62
3.5	0.68

In vitro* antioxidant activity of aqueous leaf extract of *Centella asiatica

Reducing power activity

Reducing power activity is often used to evaluate the ability of natural antioxidant to donate electron. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing power activity of *Centella asiatica* increased consistently with the increase in the volume of extract from 0.5 mg/ml to 3.5 mg/ml.When compared with the showed higher absorbance. It is known further that the reducing power activity of *Centella asiatica* was due to the power of presence of compound such as ascorbic acid (Table.3 & Fig.3).

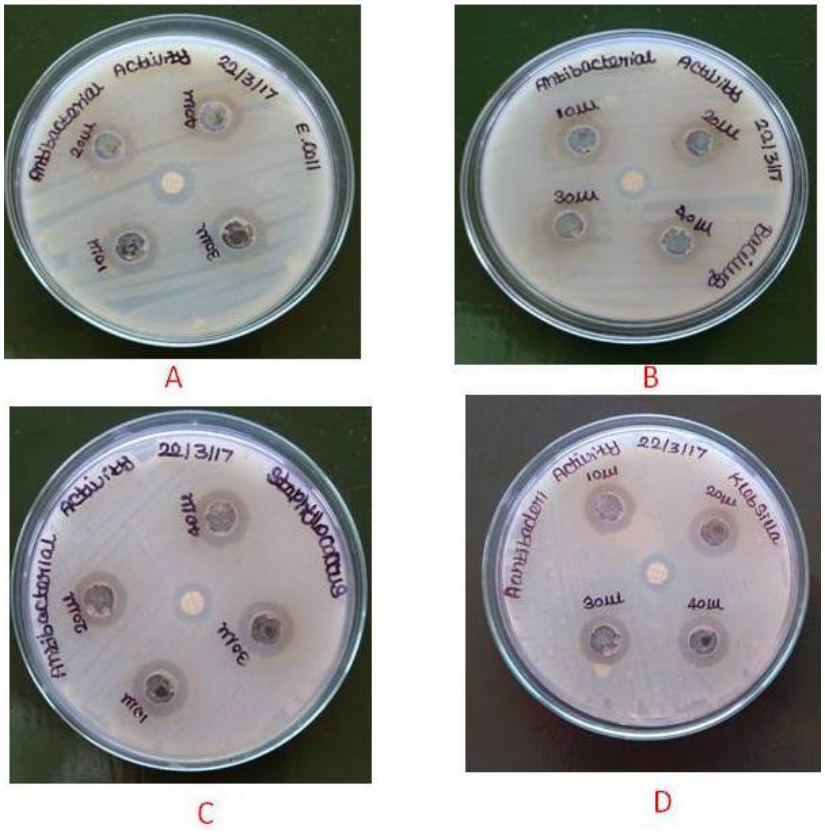

Figure 2 Antibacterial activity of aqueous leaf extract of against (a) *E.coli* (b) *Bacillus* (c) *Staphylococcus* (d) *Klebsiella*

Biosynthesis of AgNPs from Centella asiatica

The aqueous extract of centella asiatica was used as reducing agent for the synthesis of AgNPs using 1mM concentration of AgNO3. The crude aqueous extract was light brown color however after addition of AgNO3 the color of reaction mixture turned dark brown color which indicated the formation of AgNPs after 24h incubation period. The synthesized AgNPs by reduction of silver nitrate during exposure to leaves aqueous extract was confirmed by UV-Vis spectral analysis, the surface Plasmon resonance peak observed at 430nm.

Characterization

UV-Visible Absorption Spectra of AgNPs

The configuration of metal nanoparticles by reduction of the aqueous metal ions during exposure of *Centella asiatica* leaf extract was simply followed by UV–Vis spectroscopy (UV-1601 pcshimadzu spectrophotometer). UV-Vis absorption spectrum of silver nanoparticles in the presence of Ag/leaf extract is shown in (fig.2). The Surface Plasmon band in the silver nanoparticles solution remains close to 430 nm throughout the reaction period, suggesting that the nanoparticles were dispersed in the aqueous solution with no evidence for aggregation in UV-Vis absorption spectrum.

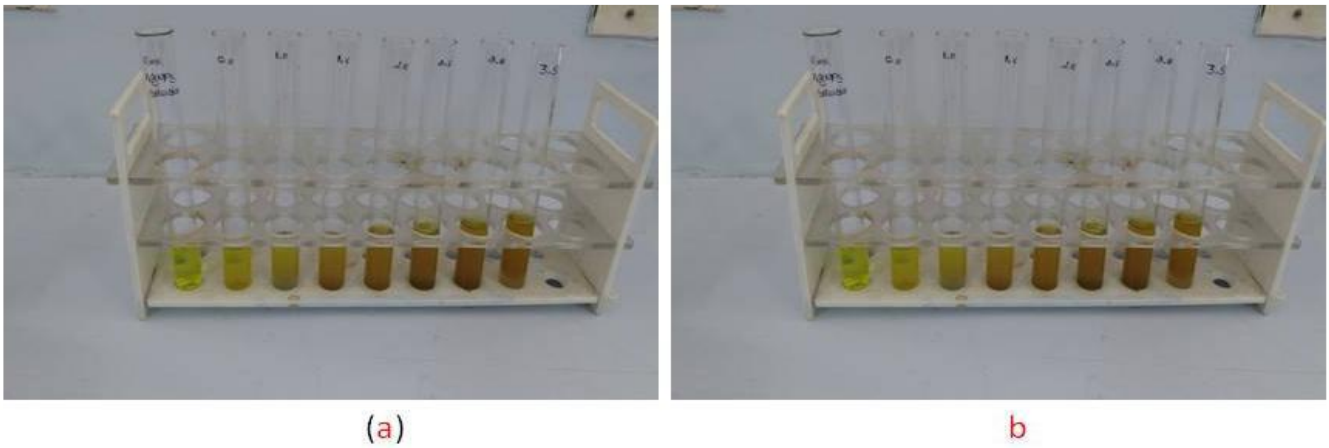

Figure 3 Reducing power activity of aqueous solution of Centella asiatica (a) Before, (b) After inoculation

Figure 4 Colour change of *Centella asiatica* leaf extract containing AgNPs before and after the synthesis UV-Visible Absorption Spectra

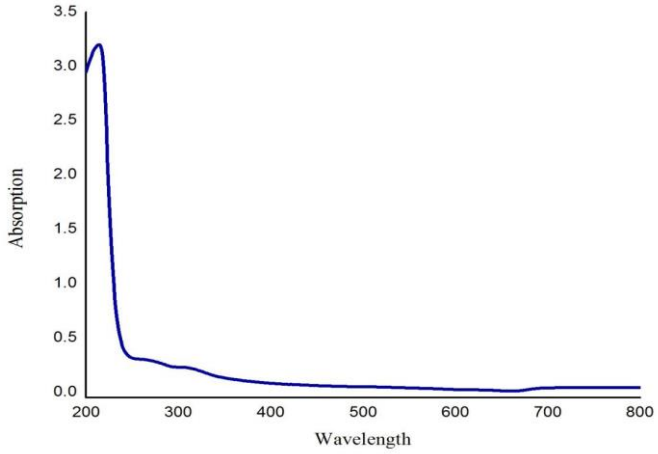

Figure 5 UV absorption of biogenic AgNPs showing surface plasmon peak at 430 nm

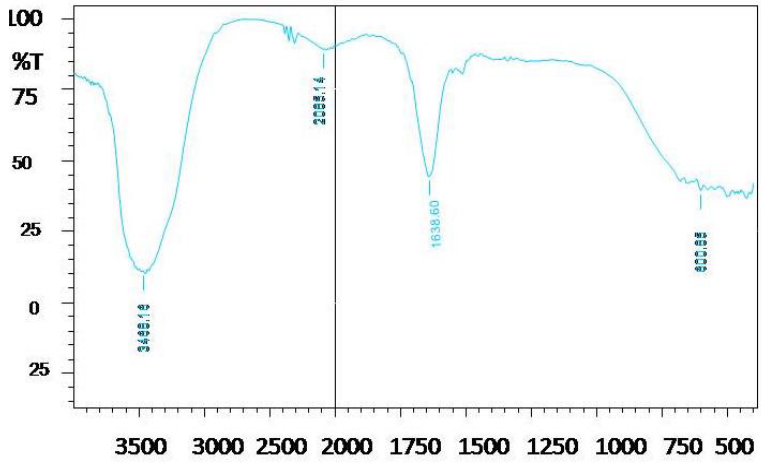

Figure 6 FTIR spectrum of *Centella asiatica* mediated bioinspired AgNPs

Fourier Transmission Infra-Red Spectroscopy (FTIR)

FTIR measurements were carried out to recognize the possible biomolecules in the leaf extract responsible for the reduction of AgNO3-ions (Figure - 4). The leaf of *Centella asiatica* contains 21% *Chavicol* (15) and the polyphenols and carboxylic compounds of *Centella asiatica* represents the FTIR spectrum of *Centella asiatica* leaf extract which shows prominent absorption bands at 1657 cm-1, 1467 cm-1 and 3420 cm-1. The shoulder at 1657 **c**m–1 is characteristic of carbonyl stretch vibrations from carboxylic acid and phenols, while the stretch at 1467 arises due to the C-O stretching and O-H deformation possibly from the acid groups present in the *Centella asiatica* leaf extract. The broad stretching at 3286 cm-1arises due to the free O-H groups present in the phenols.

Figure - 6, represents the FTIR spectrum of the *Centella asiatica* leaf extract reduced silver with the absorption bands at 1467 cm-1, 1657 cm-1 and 3240 cm-1. The shift in the carbonyl stretch frequency (1657 cm-1) to lower wave numbers (1657 cm-1) followed by the disappearance of the 1605 cm-1 resonance may be due to its binding with the silver nanoparticles surface. The shift in the C-O stretching and O-H deformation frequency (1467 cm-1) to lower wave numbers (1467 cm- 1) followed by the disappearance of the 1467 cm-1 resonance indicate the facilitation of the binding of O-H group of phenols with the silver nanoparticle surface. In addition to above supportive evidence the 3286 cm-1 feature shifts to 2895 cm-1 due to the binding of the hydroxyl group with silver nanoparticle surface.

XRD *(X-Ray Diffraction Measurement)*

The formation of silver nanoparticles synthesized using leaf extract was further supported by X-ray diffraction (XRD) measurements (Figure - 5). It was observed that *Centella asiatica* leaf extract /Ag showed strong reflections at 2θ of 19.12° and 23.23°.For Ag/ leaf extract, the XRD peaks at 2θ of 37.91°, 43.71°, 64.6° and 76.98° were characteristics to the (111), (200), (220), and (311) planes of the face-centered cubic (FCC) of Ag NPs, respectively. The peaks showed that the main composition of nanoparticles was silver and no obvious other peaks present as impurities were found in the XRD patterns. Therefore, this gives clear evidence for the presence of Ag NPs in the Ag/leaf extract. The (200), (220), (311) Bragg reflections are weak and considerably broadened relative to the intense (111) reflection. This interesting feature indicates that silver nano-crystals are in the film are predominantly (111)- oriented. Where 0.9 is the shape factor, generally taken for a cubic system, λ is the X-ray source wavelength, typically 1.54 A, β is the full width at half the maximum intensity (FWHM) in radians, and θ is the Bragg angle. Using the above formula the crystallite size was calculated to be ~13nm.

SEM *(Scanning Electron Microscope)*

Centella asiatica silver nanoparticles were subjected for the SEM analysis in which the SEM photos of silver nanoparticles and SEM photograph of *Centella asiatica* silver nanoparticles clearly showed that in the room temperature synthesized samples (*Centella asiatica*), the size (diameter) of the nanoparticles lie between 30 - 50 nm. The average size of the nanoparticles is ~ 100 nm, and the shapes were spherical and cubic.

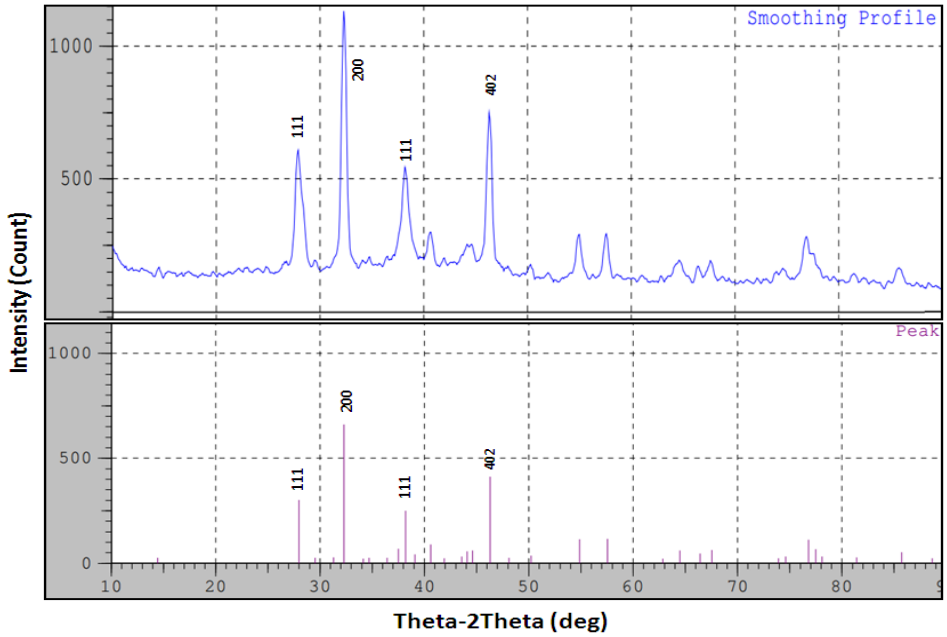

Figure 7 XRD analysis of *Centella asiatica* mediated bioinspired AgNPs

EDX analysis

The occurrence of the elemental silver can be identified by the EDX analysis, which indicated the reduction in silver ions to silver element in the reaction mixture. The EDX spectrum illustrated the presence of strong metallic Ag signals. It confirmed the elemental constituents of silver (87.65 %), chlorine (6.49 %) and Oxygen (5.86 %), respectively. The most principal sharp signal was observed at ˜3 keV for silver, which is distinctive for the absorption of crystalline nature of biosynthesized AgNPs.

Discussion

The present study reports the simple and ecofriendly approach for biosynthesis of silver nanoparticles (AgNPs) using aqueous callus extract as reducing agent for the first time. The formation of AgNPs was initially confirmed by characteristic surface plasmon resonance (SPR) peak 453 nm by UV–Visible spectroscopy. FTIR spectrum shows different functional groups which probably involved in the synthesis and stabilization of AgNPs. TEM analysisdetermined the well-dispersed AgNPs with roughly spherical shape and size ranging 5–40 nm. XRD patterns revealed the crystalline nature of AgNPs with face-centered cubic (fcc) lattice. The synthesized

AgNPs were found to have strong inhibitory activity against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa (Vasudeva *et al.,* 2014).

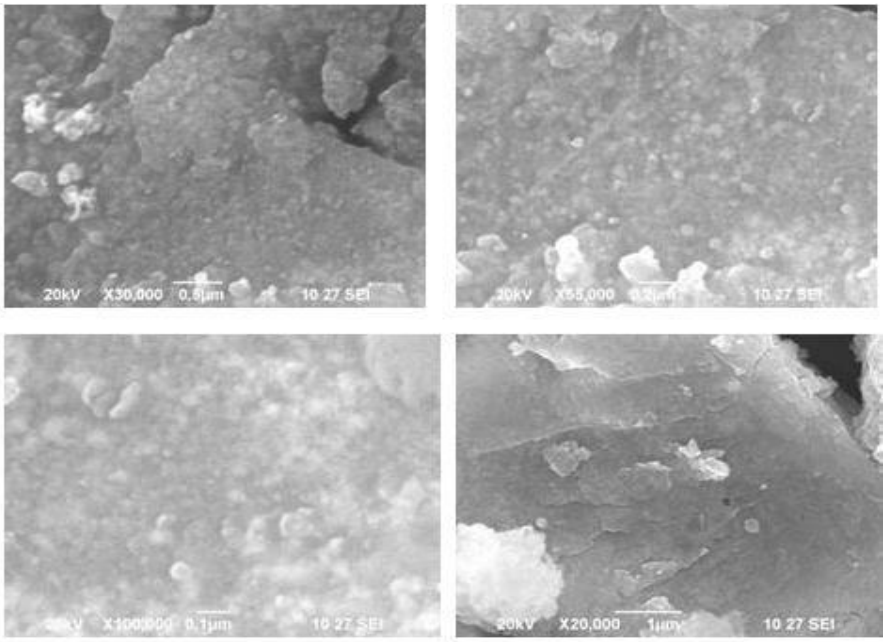

Figure 8 SEM micrograph of silver nanoparticles formed after reaction of *Centella asiatica* extract with 1mM AgNO3

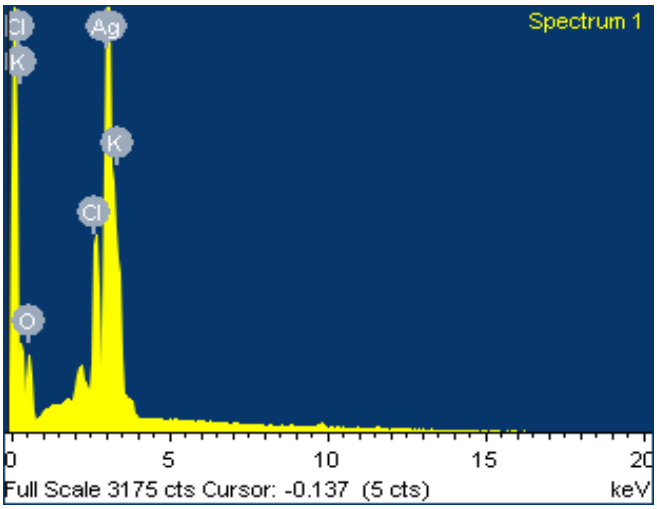

Figure 9 Energy dispersive X-ray spectrometers *Centella asiatica*

AgNO3 (2Mm) solution changed from yellowish green to brown, the final colour appeared gradually with time. The entire reaction mixture turned to brown colour after 12hrs of reaction and exhibits as absorbance (Shiying *et al*., 2007) and due to different shape of alone spherical or roughly spherical Ag nano particles. In the present study ,the conical flask with leaf broth (*Centella asiatica*) were mixed with silver nitrate solution before action the silver containing solution in greenish colour and after change to a brownish colour on the completion of the reaction. In the present study, the leaf extract of *Centella asiatica* showed a good antibacterial activity against Bacillus sp, Staphylococcus sp, Klebsiella sp, and E.coli. The result of phytochemical analysis show that phenols, tannins, saponins, flavanoids are present in the leaf of *Centella asiatica*. The reducing power scavenging activity of the plant of synthesized silver nano particles showed potential antioxidant activity.

4. CONCLUSION

The present study, a simple and eco-friendly, biosynthesis of silver nanoparticles using the plant leaves of *Centella asiatica*. In this study, AgNPs were synthesized extra cellular by *Centella asiatica* at room temperature. The AgNPs were quite stable without using any toxic chemicals as capping agents. The spherical AgNPs ranged in size from 2 nm, and showed promising broad-spectrum antimicrobial activity. The ability to synthesize AgNPs as potential antimicrobial agents using *Centella asiatica* is highly promising for the green, sustainable production of nano-metals, and this enhances its widespread application as an important strategy. Although Ag NPs themselves do not have any antimicrobial activity, they may act as drug carriers.

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DISCLOSURE STATEMENT

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Conflict of Interest: The authors declare that there are no conflicts of interests.

Data and materials availability: All data associated with this study are present in the paper.

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